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dsRNA-mediated gene silencing in cultured *Drosophila* cells: a tissue culture model for the analysis of RNA interference

Natasha J. Caplen [a], Jamie Fleenor [b], Andrew Fire [b], Richard A. Morgan [a,*]

[a] *Clinical Gene Therapy Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA*
[b] *Department of Embryology, Carnegie Institution of Washington, Baltimore, MD, USA*

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Abstract

RNA interference (RNAi) is a form of post-transcriptional gene silencing that has been described in a number of plant, nematode, protozoan, and invertebrate species. RNAi is characterized by a number of features: induction by double stranded RNA (dsRNA), a high degree of specificity, remarkable potency and spread across cell boundaries, and a sustained down-regulation of the target gene. Previous studies of RNAi have examined this effect in whole organisms or in extracts thereof; we have now examined the induction of RNAi in tissue culture. A screen of mammalian cells from three different species showed no evidence for the specific down-regulation of gene expression by dsRNA. By contrast, RNAi was observed in *Drosophila* Schneider 2 (S2) cells. Green fluorescent protein (GFP) expression in S2 cells was inhibited in a dose-dependent manner by transfection of dsRNA corresponding to *gfp* when GFP was expressed either transiently or stably. This effect was structure- and sequence-specific in that: (1) little or no effect was seen when antisense (or sense) RNA was transfected; (2) an unrelated dsRNA did not reduce GFP expression; and (3) dsRNA corresponding to *gfp* had no effect on the expression of an unrelated target transgene. This invertebrate tissue culture model should allow facile assays for loss of function in a well-defined cellular system and facilitate further understanding of the mechanism of RNAi and the genes involved in this process.

Keywords: Post-transcriptional gene silencing; RNAi; S2 cells

1. Introduction

The repression of exogenous or selfish genetic material from viruses and transposons is essential for the maintenance of organismal viability. Cellular defense mechanisms include a variety of transcriptional and post-transcriptional surveillance processes (Wolffe and Matzke, 1999). A substantial component of post-transcriptional surveillance mechanisms is triggered by double stranded RNA (dsRNA). Double stranded RNA has been shown to induce post-transcriptional gene silencing (PTGS) in a number of species including nematodes, planaria, trypanosomes, hydra, zebrafish, *Drosophila*, and mouse (Fire et al., 1998; Kennerdell and Carthew, 1998; Montgomery et al., 1998; Ngo et al., 1998; Timmons and Fire, 1998; Bahramian and Zarbl, 1999; Lohmann et al., 1999; Misquitta and Paterson, 1999; Sanchez Alvarado and Newmark, 1999; Wargelius et al., 1999; Li et al., 2000; Wianny and Zernicka-Goetz, 2000). In several cases dsRNA has been shown to induce a degradation response in which single stranded RNA complementary to the dsRNA trigger is rapidly degraded (Montgomery et al., 1998; Bosher et al., 1999).

To date, the majority of studies utilizing dsRNA-mediated gene silencing have introduced the dsRNA

Abbreviations: βgal, β-galactosidase; bp, base pair(s); BSA, bovine serum albumin; *cat*, gene encoding CAT; CAT, chloramphenicol acetyltransferase; cDNA, DNA complementary to RNA; CM, conditioned medium; CMV, cytomegalovirus; CPRG, chlorophenol red-β-D-galactopyranoside; DMSO, dimethylsulfoxide; ds, double stranded; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence activated cell analysis; FBS, fetal bovine serum; G418, Genticin; *gfp*, gene encoding GFP; GFP, green fluorescent protein; HPLC, high performance liquid chromatography; kb, kilobase (s); *lacZ*, gene encoding βgal; nt, nucleotide(s); p, plasmid; PCR, polymerase chain reaction; PKR, dsRNA-dependent protein kinase; PTGS, post-transcriptional gene silencing; RNAi, RNA interference; S2, *Drosophila* Schneider 2 cells; ss, single stranded.

* Corresponding author. Tel.: +1-301-402-1830; fax: +1-301-402-1921.
E-mail address: rmorgan@nhgri.nih.gov (R.A. Morgan)

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into whole organisms, in particular early embryos. This application has been a powerful reverse genetics tool, particularly for the study of developmentally regulated genes (Kennerdell and Carthew, 1998; Fay et al., 1999; Kwon et al., 1999; Misquitta and Paterson, 1999), however, these systems have inherent difficulties in generating sufficiently uniform material to study the mechanisms that induce this response. Recently, Tuschl and coworkers described the inhibition of gene expression by dsRNA in vitro using *Drosophila* embryo cell lysates (Tuschl et al., 1999). In this same study, rabbit reticulocyte cell extracts generated a rapid but non-specific decrease in mRNA. The failure of dsRNA to produce a specific inhibition of gene expression in the presence of rabbit reticulocyte lysates is consistent with previous observations of a prevalent and non-specific response to dsRNA in mammalian cells. One component of the response to dsRNA in mammalian cells is mediated by the dsRNA-dependent protein kinase (PKR) which phosphorylates and inactivates the translation factor eIF2α, leading to a generalized suppression of protein synthesis, and in some cases apoptosis (Clemens and Elia, 1997).

We have now screened commonly used invertebrate and vertebrate cell lines with the aim of determining whether dsRNA-mediated gene silencing can be induced in cell culture systems. No evidence of gene-specific dsRNA-mediated gene silencing was detected in three mammalian cell lines, but a robust effect of dsRNA was seen in *Drosophila* Schneider 2 (S2) cells (Schneider and Blumenthal, 1978). Invertebrate cell culture systems, particularly those from *Drosophila melanogaster*, are well established and have become a valuable tool in analyzing biological function (Schneider and Blumenthal, 1978; Cherbas et al., 1994). Studies in this tissue culture model should significantly aid in our understanding of PTGS, and allow the rapid screening of suitable target sequences and dsRNA formulations.

2. Materials and methods

2.1. Plasmids and dsRNA

Using pEGFP-C (Clontech) as DNA template, the *gfp* gene was PCR amplified using the following primers: 5′ GGGGATCCATGGTGAGCAAG 3′ and 5′ GGCTGCAGTTATTACTTGTACAG 3′ to add *Bam*HI and *Pst*I restriction sites to the 5′ and 3′ ends of the *gfp* gene, respectively. Using pOR13CAT (Stratagene, La Jolla, CA) as DNA template, the *cat* gene was PCR amplified using the following primers: 5′ GGGGATCCATGGAGAAAAAAATC 3′ and 5′ GGCTGCAGTTATTACGCCCCG 3′ to add *Bam*HI and *Pst*I restriction sites to the 5′ and 3′ ends of the *cat* gene, respectively. In both cases, the appropriately sized PCR fragments were subcloned into pPCR (Stratagene) and *Bam*HI–*Pst*I *gfp* and *cat* fragments prepared for insertion into *Bam*HI and *Pst*I restricted pRActHAdh (a *Drosophila* expression vector containing the *Drosophila* constitutive *Actin 5C* promoter), generating pAct.GFP and pAct.CAT, respectively. The selectable marker plasmid p8HCO carries a methotrexate-resistance gene; pPC4 expresses a mutant RNA polymerase II conferring resistance to α-amanitin (Thomas and Elgin, 1988). The pCMVβ plasmid expressed β-galactosidase (βgal) under the control of the CMV promoter (Clontech, Palo Alto, CA), pGL3 (Promega, Madison, WI) expresses luciferase under the control of the SV40 early enhancer/promoter. Double stranded RNA was generated corresponding to the *gfp* gene (entire coding sequence, 717 bp) and the βgal (*lacZ*) gene (nt 1945 to 2774 of the 3066 nt coding region) using previously described methods (Fire et al., 1998). Sense and antisense RNA oligonucleotides (78 mers) corresponding to *gfp* (nt 276 to 354 of the 717 bp coding region) and *cat* (nt 313 to 392 of the 675 bp coding region) were chemically synthesized using standard methods and HPLC purification (Xeragon AG, Zurich, Switzerland).

2.2. Cell culture and nucleic acid transfections

S2 cells were grown in DES® Medium (Invitrogen, Carlsbard, CA) supplemented with 10% fetal bovine serum (FBS) (Gemini BioProducts Inc., Calabasas, CA). Cells were passaged every two to three days to maintain exponential growth. S2 cells were transfected using either the cationic lipid CellFectin (Life Technologies, Gaithersburg, MD), or DOTAP (Roche Biochemicals, Indianapolis, IN) using an adaptation of the manufacturer's protocol. Briefly, cells were seeded and allowed to settle overnight, nucleic acid (plasmid and/or dsRNA) was complexed with lipid at a weight to weight ratio of 1:6 in DES® medium without supplementation. The complex was incubated at room temperature for 15 min and then added to cells from which normal growth medium had been removed. After overnight incubation an equal volume of DES® medium plus 20% FBS was added to the cell/lipoplex mixture. Where applicable, pBluescript (pBS, Stratagene) was used as a DNA carrier in lipofections to maintain consistency in the amount of DNA transfected. To establish selected populations of S2 cells, the pAct.GFP (10 μg) or pAct.CAT (10 μg) plasmids were co-transfected with 1–3 μg of p8HCO or pPC4 using DOTAP cationic lipid. Cells were selected using 90 ng/ml methotrexate (Sigma, St. Louis, MO) or 5 μg/ml α-amanitin (Sigma) for a minimum of five weeks.

293 (human embryonic kidney cells) (Graham and Prevec, 1992) and NIH-3T3 (mouse fibroblast, ATCC:CRL1658) were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS and

penicillin/streptomycin (Life Technologies). BHK-21 (baby hamster kidney, ATCC Cat #CCL-10) cells were grown in complete BHK medium (Glasgow MEM, 2 mM glutamine, 10% tryptose phosphate broth, 10 mM HEPES, and 5% FBS). NIH-3T3 cells were transduced with an ecotropic retroviral vector expressing the *lacZ* and the *neomycin phosphotransferase* genes and selected with 1 mg/ml G418 to generate a permanently expressing population. Transfections were conducted as above, replacing DES medium with low serum OptiMEM medium (Life Technologies) for the formation of the lipoplex and lipofection.

2.3. RNA analysis

Total RNA was isolated from S2 cells using either NP40 lysis, SDS/Proteinase K digestion, and phenol/chloroform extraction or by guanidine thiocynate extraction (Ambion, Austin, TX). Poly(A^+) RNA was selected using Oligo dT cellulose (Ambion) and analyzed by electrophoresis (1.2% agarose, 1 × MOPS, 5.0% formaldehyde), Northern blot transfer, and hybridization (Ambion) at 42°C with a ^{32}P-labelled random-primed probe for *gfp*. Filters were washed at high stringency and subjected to autoradiography. To standardize for transfection efficiency, RNA integrity, and loading variations, filters were striped and rehybridized with a cDNA probe corresponding to *cat* and/or a cDNA probe corresponding to *Drosophila gapdh-1* (nucleotides 617–1483) generated by RT-PCR (Superscript, Life Technologies) of S2 mRNA (forward primer 5′ CCA GAA GAT CAC CGT GTT C 3′ and reverse primer 5′ CCC TTG CGG ATT ATG CAA C 3′). An RNA ladder was used to estimate transcript sizes (Ambion).

2.4. Transgene expression

Green fluorescent protein expression was assessed using fluorescence activated cell analysis (FACS) (FacsCaliber, Becton Dickinson, San Jose, CA). S2 cells transfected with pAct.CAT, S2/CAT/8HCO, or S2/CAT/PC4 cells were used to gate for forward scatter and side scatter; 10 000 events were captured per sample. The percentage of GFP positive cells was determined by gating against pAct.CAT transfected cells or S2/CAT/8HCO or S2/CAT/PC4 cells, the geometric mean fluorescence was used as a measure of the relative intensity of fluorescence. Chloramphenicol acetyl transferase expression was assessed using an ELISA assay (Roche Biochemicals). β-Galactosidase expression was assessed by colorimetric assay using CPRG as chromogenic substrate (Felgner et al., 1994; Caplen et al., 1995). Total protein was determined using the Bradford micro-assay protocol (Bio-Rad, Hercules, CA). Absorption readings (A_{595}) were converted to absolute amounts using a bovine serum albumin (Sigma) (0.625–10 μg) standard curve after subtraction of background values, numbers were expressed as mean ± the standard error of the mean (SEM). For statistical analyses StatView 5 was used to apply an unpaired *t*-test (SAS Institute Inc., San Francisco, CA); the null hypothesis was rejected at $P \geq 0.05$.

3. Results

3.1. dsRNA-mediated gene silencing in Drosophila cells in culture: (i) the effect on transient transgene expression

The aim of this study was to ascertain whether dsRNA-mediated gene silencing could be induced in tissue culture cells. Double stranded RNA was generated corresponding to two segments: one from *green fluorescent protein* (*gfp*) and one from *β-galactosidase* (*lacZ*). As a model invertebrate tissue culture line we use *Drosophila* S2 cells (Schneider, 1972). Two *Drosophila* expression plasmids were constructed, the first expressed GFP (pAct.GFP), the second, a plasmid control, expressed chloramphenicol acetyltransferase or CAT (pAct.CAT). We assessed the effect of dsRNA on transgene expression from transiently transfected pAct.GFP. A representative FACS analysis of transfected S2 cells 72 h after initiation of transfection is shown in Fig. 1A–D. In positive controls, approximately 40% of S2 cells were positive for GFP (Fig. 1B) following transfection with pAct.GFP. The co-transfection of dsRNA corresponding to *lacZ* altered neither the number of cells expressing GFP nor the intensity of GFP fluorescence (Fig. 1C). In contrast, the co-administration of *gfp* dsRNA dramatically reduced both the numbers of cells expressing GFP and the relative intensity of GFP fluorescence (Fig. 1D).

To analyze this change in *gfp* gene expression at an RNA level, cytoplasmic poly(A^+) RNA was purified by standard methods and the mRNA subjected to Northern analysis using a cDNA probe corresponding to *gfp* (Fig. 1E). As a positive control, filters were then rehybridized with probes corresponding to *cat* and *Drosophila gapdh-1*. These experiments showed a dramatic and specific decrease in *gfp* mRNA levels in response to co-transfection with *gfp* dsRNA.

3.2. dsRNA-mediated gene silencing in Drosophila cells in culture: (ii) the effect on stable transgene expression

The transient model system described above cannot exclude that the inhibition of gene expression is due to interference in the transfection process rather than a direct effect on gene expression. We thus carried out a series of experiments in which RNAs were transfected into cells already expressing a transgene. We first pro-

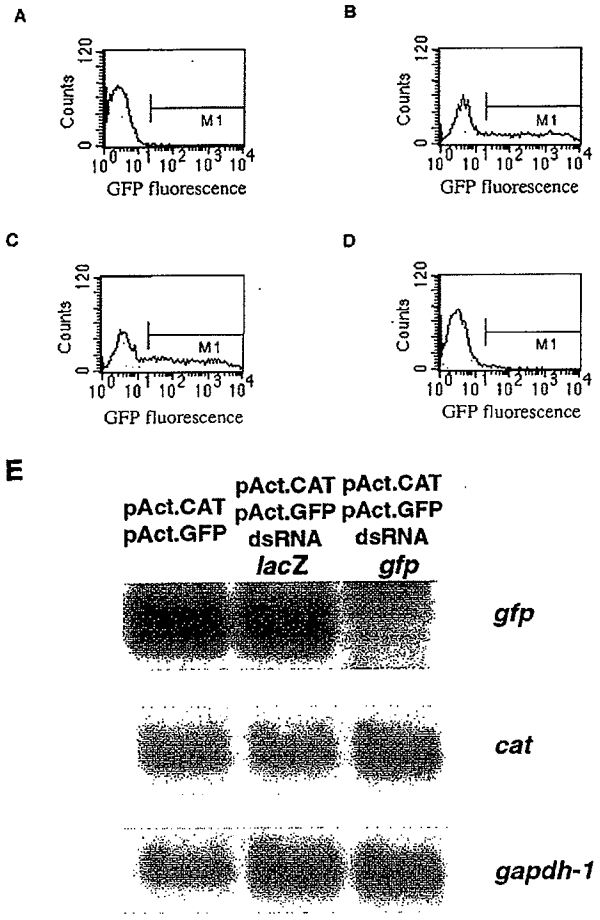

Fig. 1. dsRNA-mediated gene silencing in a tissue culture system. Representative FACS analysis of S2 cells transiently transfected with the *Drosophila* expression plasmids and dsRNA (5×10^6 cells initially plated). Transfections were as follows: (A) pAct.CAT (20 μg) (M1 = 0.06%, geometric mean fluorescence: all cells 2.49, M1 cells 30.92); (B) pAct.GFP (20 μg) (M1 = 41.31%, geometric mean fluorescence: all cells 27.23, M1 cells 347.87); (C) pAct.GFP (20 μg) and *lacZ* dsRNA (5 μg) (M1 = 43.89%, geometric mean fluorescence: all cells 26.46, M1 cells 258.70); (D) pAct.GFP (20 μg) and *gfp* dsRNA (5 μg) (M1 = 1.35%, geometric mean fluorescence: all cells 3.19, M1 cells 37.83). M1 indicates the gating for GFP positive cells. (E) Northern analysis of poly(A^+) RNA (approximately 1.5 μg of each sample) purified from S2 cells transiently transfected with *Drosophila* expression plasmids and dsRNA.

duced lines of S2 cells which stably express GFP by co-transfecting S2 cells with the pAct.GFP plasmid and a selectable marker plasmid either conferring resistance to methotrexate (p8HCO) or α-amanitin (pPC4). After several weeks of selection, two populations of cells expressing GFP were generated (S2/GFP/8HCO and S2/GFP/PC4 cells). These cells were then subjected to transfection with dsRNA (Fig. 2A and D). Introduction of *gfp* dsRNA decreased the number of cells expressing GFP by 60–70% in S2/GFP/8HCO cells and 80% in S2/GFP/PC4 cells (Fig. 2C and F). There was also a decrease in the relative intensity of GFP fluorescence (see legend to Fig. 2). Transfection of *lacZ* dsRNA showed no effect on GFP expression either in terms of total number of positive cells or intensity of GFP fluorescence (Fig. 2B and E). The effects of dsRNA could also be seen in these experiments as a decrease in steady-state levels of *gfp* mRNA transcripts (Fig. 2G). Control experiments with a non-homologous gene (*gapdh-1*) or an irrelevant dsRNA (*lacZ*) demonstrated that this effect on mRNA levels was sequence-specific.

3.3. *dsRNA-mediated gene silencing in tissue culture is sequence-specific, structure-specific and dose-dependent*

RNA interference in whole animal systems and *Drosophila* cell extracts is dependent on the structure and dose of the interfering RNA (Fire et al., 1998; Kennerdell and Carthew, 1998). To assess this in S2 cells we used both the transient and stable transgene expression models to compare the effect of single and double stranded RNA molecules (Fig. 3). No interference was seen with purified sense and antisense RNAs. In contrast, dsRNA *gfp* molecules induced a statistically significant decrease in gene expression in both the transient and stable expression systems (S2 cells transfected with pAct.GFP and pAct.CAT vs. S2 cells transfected with pAct.GFP and pAct.CAT co-transfected with *gfp* dsRNA, $P = 0.04$; S2/GFP/PC4 cells vs. S2/GFP/PC4 cells transfected with *gfp* dsRNA, $P = 0.0003$).

We also assessed whether RNA interference in these cells was dose-dependent (Fig. 4). At no dose did *lacZ* dsRNA induce any significant reduction in GFP expression. By contrast, as little as 0.1 μg of *gfp* dsRNA induced a significant decrease ($P = 0.02$) in gene expression in the transient model (Fig. 4A), with further decreases as the doses of dsRNA increased (0.5 μg *gfp* dsRNA $P = 0.005$, 1.0 μg *gfp* dsRNA $P = 0.003$, 5.0 μg *gfp* dsRNA $P = 0.002$). Cells stably expressing GFP required more *gfp* dsRNA to induce a statistically significant decrease in transgene expression, however, this inhibition of GFP expression was also dose-dependent (Fig. 4B and C) (percentage GFP positive cells, 1.0 μg *gfp* dsRNA $P = 0.03$, 5.0 μg *gfp* dsRNA $P = 0.01$; relative geometric mean fluorescence of all cells sampled, 1.0 μg *gfp* dsRNA $P = 0.045$, 5.0 μg *gfp* dsRNA $P = 0.01$). Similar structure-specific, sequence-specific and dose responses were observed using S2/GFP/8HCO cells (data not shown).

In all of these studies no effect on CAT expression was seen following transient co-transfection with pAct.CAT, indicating the specificity of this inhibition and total protein levels did not differ significantly in either the transient or stable cells, suggesting that there was no overt toxicity or down-regulation in protein expression (representative data shown in Table 1).

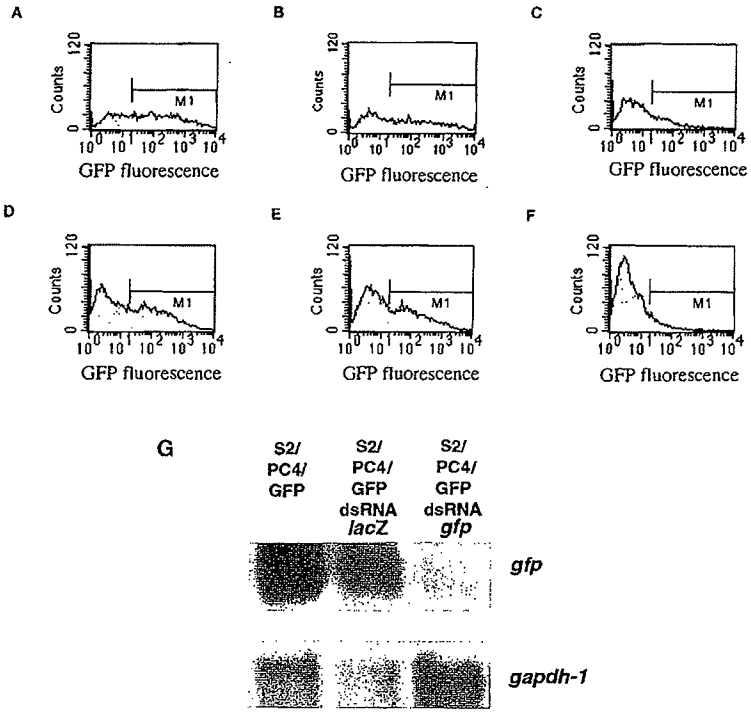

Fig. 2. dsRNA-mediated gene silencing of a stably expressed transgene target in S2 cells. Representative FACS analysis of S2/GFP/8HCO and S2/GFP/PC4 cells (5×10^6 cells initially plated) transfected with *lacZ* or *gfp* dsRNA; 5 μg of dsRNA complexed with 30 μg cationic lipid was transfected on three occasions approximately 48 h apart, cells were assayed six days after initiation of the first transfection. (A) Untransfected S2/GFP/8HCO cells (M1 = 64.03%, geometric mean fluorescence: all cells 54.54, M1 cells 192.88). (B) S2/GFP/8HCO cells transfected with *lacZ* dsRNA (M1 = 53.13%, geometric mean fluorescence: all cells 36.62, M1 cells 200.44). (C) S2/GFP/8HCO cells transfected with *gfp* dsRNA (M1 = 19.07%, geometric mean fluorescence: all cells 7.38, M1 cells 54.39). Reference control cells were S2/CAT/8HCO cells (M1 = 0.01%, geometric mean fluorescence: all cells 1.88, M1 cells 30.23). (D) Untransfected S2/GFP/PC4 cells (M1 = 42.12%, geometric mean fluorescence: all cells 16.02, M1 cells 115.96). (E) S2/GFP/PC4 cells transfected with *lacZ* dsRNA (M1 = 40.21%, geometric mean fluorescence: all cells 17.59, M1 cells 115.10. (F) S2/GFP/PC4 cells transfected with *gfp* dsRNA (M1 = 7.32%, geometric mean fluorescence: all cells 4.41, M1 cells 43.37). Reference control cells were S2/CAT/PC4 cells (M1 = 0.26%, geometric mean fluorescence: all cells 2.00, M1 cells 76.22). M1 indicates the gating for GFP positive cells. (G) Northern analysis of poly(A^+) RNA (approximately 1.5 μg of each sample) purified from S2/GFP/PC4 cells transfected with *lacZ* or *gfp* dsRNA.

3.4. dsRNA-mediated gene silencing in tissue culture using RNA oligonucleotides

Previous in vitro and in vivo studies of RNAi have shown a relationship between the length of dsRNA and the magnitude of the reduction in gene expression, with longer dsRNA molecules (over approximately 100 in vivo and over 500 in vitro) performing more effectively than smaller molecules (Ngo et al., 1998; Tuschl et al., 1999). To assess if shorter dsRNA molecules were effective in S2 cells, we used dsRNA generated from RNA oligomers of 78 nucleotides in length corresponding to the central portion of both the *gfp* and the *cat* coding regions. Fig. 5 shows the inhibition of both GFP and CAT expression when the corresponding dsRNA molecules (1 μg) are co-transfected with both the GFP and CAT expressing plasmids pAct.GFP and pAct.CAT (Fig. 5A and B). Expression of the control transgene was unaffected, as were total protein levels (Fig. 5C). In addition, CAT expression was significantly decreased in cells stably expressing CAT following transfection of the 78 mer (untransfected S2/CAT/8HCO cells vs. S2/CAT/8HCO cells transfected with 1 μg *cat* dsRNA, $P < 0.0001$ and 2 μg *cat* dsRNA, $P < 0.0001$) (Fig. 5D); no consistent effect on protein levels was seen (Fig. 5E).

3.5. The transfer of RNAi between populations of S2 cells

The widespread effect of PTGS throughout an organism, when only a limited number of cells have been originally treated with the triggering dsRNA, has led to speculation of the presence of a soluble factor or factors that can spread RNAi from cell to cell (Palauqui et al., 1997; Palauqui and Balzergue, 1999). To determine if RNAi generated in one population of S2 cells can induce RNAi in a second population of S2 cells, we first induced RNAi using the transient transfection model (Fig. 6A). S2 cells were co-transfected as described above, after 24 h the medium containing the lipoplex

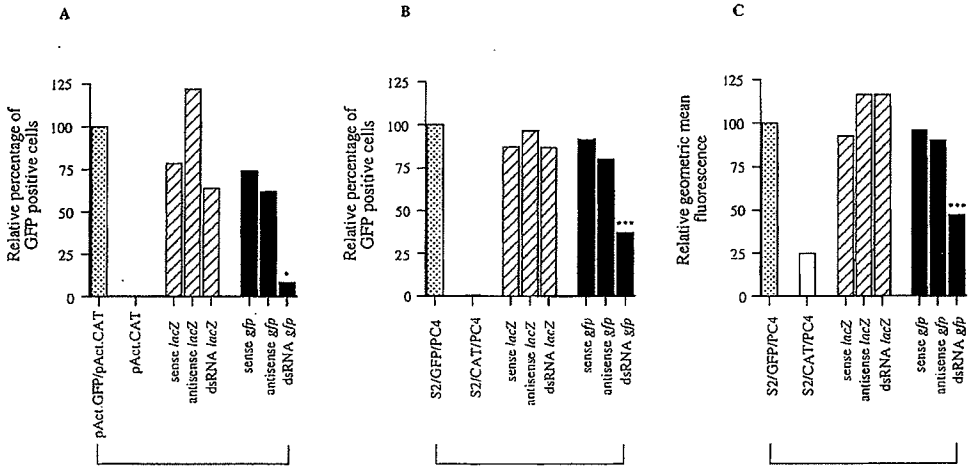

Fig. 3. GFP transgene expression is inhibited more effectively by dsRNA than antisense GFP. (A) Comparison of the effect of sense (5 μg), antisense (5 μg), and dsRNA (5 μg) molecules on GFP expression in transiently transfected S2 cells (5×10^6 cells initially plated). Cells were assayed 72 h after initiation of transfection. Data is presented as a relative percentage of GFP expressing cells standardized against S2 cells transfected with pAct.GFP (10 μg) and pAct.CAT (10 μg) only ($n=3$ for all transfection combinations). (B, C) A comparison of the effect of a single transfection of sense (5 μg), antisense (5 μg), and dsRNA (5 μg) molecules on GFP expression in S2/GFP/PC4 cells (2×10^6 cells initially plated); cells were assayed 10 days after transfection. (B) The percentage of GFP expressing cells relative to the number of GFP positive cells observed in untransfected S2/GFP/PC4 cells and (C) the relative geometric mean fluorescence of all cells sampled standardized against untransfected S2/GFP/PC4 cells ($n=3$ for all transfection combinations; * $P<0.05$, ** $P<0.01$, *** $P<0.001$).

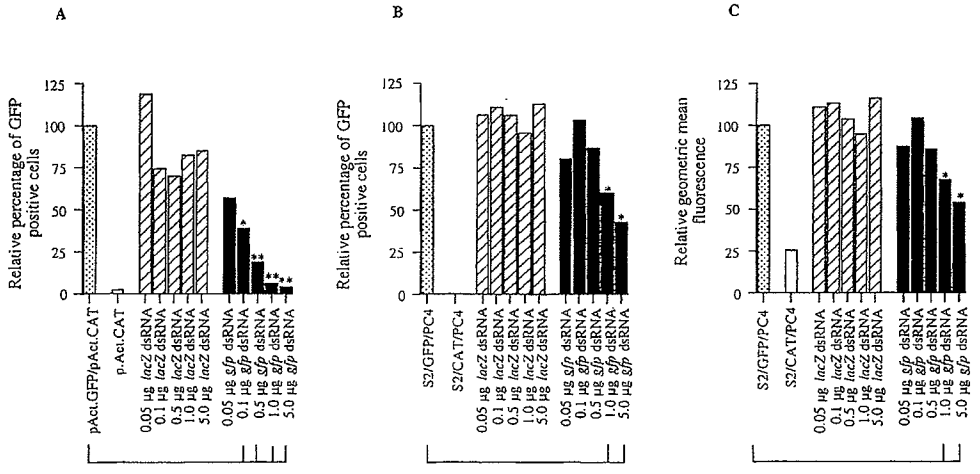

Fig. 4. Inhibition of GFP expression by dsRNA is dose-dependent. (A) The effect of increasing dsRNA doses on GFP expression in transiently transfected S2 cells (dsRNA 0.05–5 μg/5×10^6 cells initially plated); cells were assayed 72 h after initiation of transfection. Results are shown as relative percentage of GFP positive cells normalized to transfections of pAct.GFP (10 μg) and pAct.CAT (10 μg) plasmids only ($n=3$ for all transfection combinations). (B, C) The effect of increasing dsRNA doses on GFP expression in S2/GFP/PC4 cells (0.05–5 μg dsRNA/2×10^6 cells); cells were assayed 10 days after transfection. (B) The percentage of GFP expressing cells relative to the number of GFP positive cells observed in untransfected S2/GFP/PC4 cells. (C) The relative geometric mean fluorescence of all the cells sampled standardized against untransfected S2/GFP/8PC4 cells ($n=3$ for all transfection combinations; * $P<0.05$, ** $P<0.01$, *** $P<0.001$).

was removed, the cells washed and fresh medium added; supernatants were harvested 24 h later, filtered and this conditioned medium (CM) transferred to S2 cells transfected with pAct.CAT and pAct.GFP 24 h previously; cells were assayed for GFP expression seven days later (Fig. 6B and C). Conditioned medium from cells subjected to treatment with *gfp* dsRNA induced a significant decrease in GFP expression, both with respect

Table 1
Representative analysis of CAT and protein levels

Cell line	Plasmids transfected	RNA molecule transfected	CAT (ng/μg protein)[a]	Statistical comparison	Protein (μg/μl)[a]	Statistical comparison
S2	pAct.GFP pAct.CAT	–	1.99±0.56	–	7.68±0.59	–
S2	pAct.GFP pAct.CAT	*gfp* sense	3.19±1.14	$P=0.40$[b]	8.28±0.24	$P=0.40$[b]
S2	pAct.GFP pAct.CAT	*gfp* antisense	2.97±1.40	$P=0.55$[b]	8.18±0.10	$P=0.44$[b]
S2	pAct.GFP pAct.CAT	*gfp* dsRNA	1.37±0.37	$P=0.41$[b]	7.13±0.66	$P=0.57$[b]
S2/GFP/PC4		–	–	–	0.28±0.08	–
S2/GFP/PC4		*gfp* sense	–	–	0.44±0.13	$P=0.37$[c]
S2/GFP/PC4		*gfp* antisense	–	–	0.32±0.09	$P=0.77$[c]
S2/GFP/PC4		*gfp* dsRNA	–	–	0.58±0.11	$P=0.09$[c]

[a] Three independent transfections, and all assays performed in triplicate.
[b] Comparison with S2 cells transfected with pAct.GFP and pAct.CAT.
[c] Comparison with untransfected S2/GFP/PC4 cells.

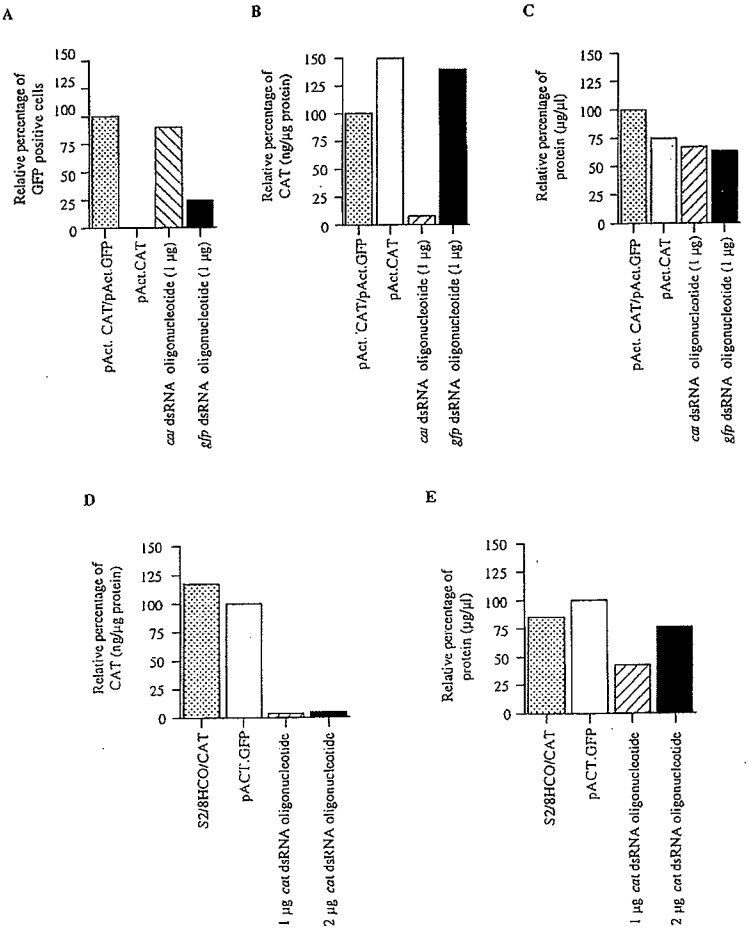

Fig. 5. RNAi can be mediated in tissue culture using RNA oligonucleotides. (A–C) The effect of co-transfecting dsRNA oligonucleotides corresponding to *gfp* or *cat* with plasmids expressing GFP or CAT. (A) The effect on GFP expression. (B) The effect on CAT expression. (C) The effect on total protein levels. (D, E) The effect of transfecting an dsRNA oligonucleotide corresponding to *cat* into S2/CAT/8HCO cells. (D) The effect on CAT expression. (E) The effect on total protein levels.

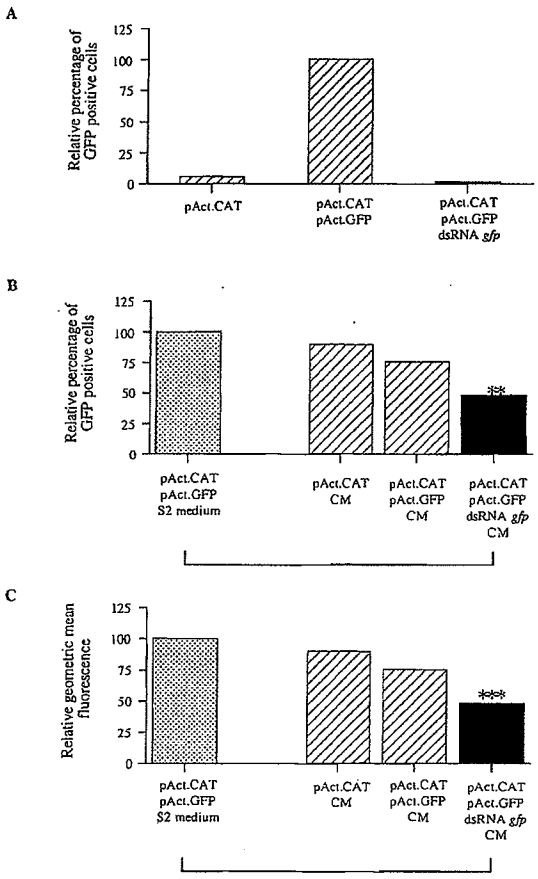

Fig. 6. RNAi can be transferred from one population of S2 cells to another. (A) The generation of RNAi in the primary population of S2 cells. S2 cells were transfected with plasmids and plasmids plus dsRNA as shown, data is presented as a relative percentage of GFP expressing cells standardized against S2 cells transfected with pAct.GFP (10 μg) and pAct.CAT (10 μg) only ($n=3$ for all transfection combinations). (B, C) Conditioned medium was transferred from the primary populations of cells (shown in A) to S2 cells transfected with pAct.CAT (10 μg) and pAct.GFP (10 μg) 24 h previously. (B) The percentage of GFP positive cells after exposure to CM for seven days normalized to cells grown in standard S2 medium. (C) The relative geometric mean fluorescence of GFP positive cells after exposure to CM for seven days normalized to cells grown in standard S2 medium ($n=3$; ** $P<0.01$, *** $P<0.001$).

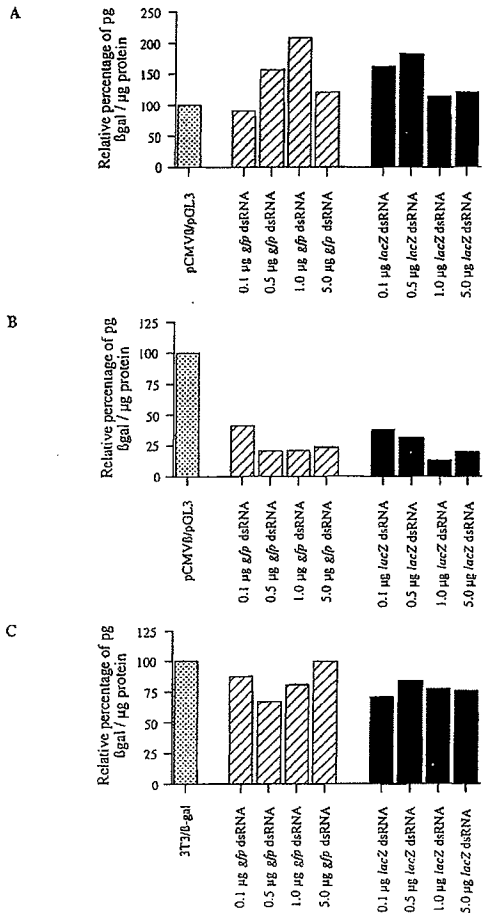

Fig. 7. Screening of mammalian cells for dsRNA-mediated gene silencing. (A) The transient co-transfection of human 293 cells. 293 cells were co-transfected with plasmids expressing βgal and luciferase and increasing amounts of *gfp* or *lacZ* dsRNA. βgal expression was assayed after 72 h and standardized against total protein, results are normalized against plasmid only transfections. (B) The transient co-transfection of BHK21 cell. BHK21 cells were co-transfected with plasmids expressing βgal and luciferase and increasing amounts of *gfp* or *lacZ* dsRNA. βgal expression was assayed after 72 h and standardized against total protein, results are normalized against plasmid only transfections. (C) The transfection of NIH-3T3 cells permanently expressing βgal. Retrovirally transduced NIH-3T3 cells expressing βgal were transfected with increasing amounts of *gfp* or *lacZ* dsRNA. βgal expression was assayed after 72 h and standardized against total protein, results are normalized against untransfected 3T3/βgal cells ($n=3$ in all cases).

to the total number of GFP positive cells ($P=0.0015$) and intensity ($P=0.0008$) in comparison with cells cultured in standard S2 medium.

3.6. Screening of mammalian cells for dsRNA-mediated gene silencing

To examine the susceptibility of mammalian tissue culture cells to RNAi we screened commonly used cell lines from three different species: human, hamster, and mouse. We used cells expressing transgenes both transiently and permanently, with βgal as the target gene and luciferase as a control transgene (Fig. 7). The *lacZ* dsRNA molecule was identical to a segment previously shown to produce effective interference after injection of *C. elegans*. An equivalent segment of dsRNA corresponding to *gfp* was used to identify non-specific dsRNA effects. Transient co-transfection of plasmid DNA and dsRNA into 293 (Fig. 6A) and BHK21 (Fig. 7B) cells resulted either in no effect at all (293 cells), or a non-specific decrease in gene expression (BHK21 cells). The

transfection of dsRNA into mouse NIH-3T3 cells transduced with a retrovirus expressing βgal induced no detectable decrease in gene expression (Fig. 7C). Luciferase expression paralleled the levels of βgal expression in all three cell lines (data not shown).

4. Discussion

A tissue culture model of RNAi would be an extremely valuable resource for understanding the biochemistry of RNAi, the genes involved in this process, and its precise role in the regulation of endogenous or exogenous gene expression. This report demonstrates that S2 *Drosophila* cells can be used as a robust model system for RNAi with all the features of in vivo RNAi tested being recapitulated in this model, including sequence and structure specificity, dose dependency and potency (Fire et al., 1998; Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999). These results are also consistent with those described in vitro (Tuschl et al., 1999), and at least in part with a similar study of RNAi in S2 cells (Hammond et al., 2000). In the study by Hammond et al., dsRNA-mediated interference of transient *lacZ* and endogenous *cyclin E* expression was induced in S2 cells. A similar, sequence-specific decline in mRNA levels was seen in both the study of Hammond and colleagues and our study, however, Hammond et al. also observed a length dependency, with dsRNAs of less than 300 nt failing to generate RNAi. This contrasts with our study, in which we observed RNAi in both our transient GFP and stable CAT S2 models following transfection of 78 mer dsRNA oligomers. This difference may be due to the fact that we targeted different genes. Additionally, the oligomers used in this study were chemically synthesized and thus may be of higher purity than those generated by in vitro transcription and gel purification. Lastly, in our report we extensively studied the dose (or concentration) dependency of effective RNAi in S2 cells, and this may be relevant when comparing dsRNA molecules of different lengths.

The transient transfection model presented here has the potential to be applied as an easy assay for optimization of RNAi (with GFP as the marker, differences between dsRNA treated and untreated populations could be quantitated within 18 h of the initiation of the transfection). This model could thus be used for each target of interest to determine optimal conditions for RNAi prior to more complex in vivo experimentation. This study also used the relatively simple and non-invasive method of cationic lipid-mediated nucleic transfer to introduce the dsRNA rather than injection, which has been the principal method used in RNAi studies in whole organisms. The transfer of dsRNA to whole organisms could be enhanced by the use of cationic lipids, protecting the dsRNA from degradation as shown in *C. elegans* (Tabara et al., 1998), as well as facilitating transfer.

The recent studies describing co-suppression and RNAi-like processes in mammalian cells (Bahramian and Zarbl, 1999; Wianny and Zernicka-Goetz, 2000) imply that, at least under specific circumstance, a PTGS- or RNAi-like mechanism might be present in mammalian cells (Bosher and Labouesse, 2000). We examined three different mammalian cells (including NIH-3T3 cells for which evidence of the induction of co-suppression has been reported; Bahramian and Zarbl, 1999) using a range of doses of dsRNA for which we had seen efficient RNAi in S2 cells, but in these cells we saw no specific effect on gene expression. Our results were consistent with the well-documented interferon-induced non-specific response of mammalian cells to dsRNA (Clemens and Elia, 1997). However, it may be that gene, cell-type or developmentally specific effects may influence the balance between specific (PTGS) and non-specific responses to dsRNA. This would need to be taken into account when considering PTGS or RNAi in mammalian cell systems.

In vivo PTGS and RNAi need only be induced locally in a small number of cells to generate gene silencing throughout the organism (Fire et al., 1998; Palauqui and Balzergue, 1999). Tuschl and coworkers have also shown in vitro that pre-incubation of dsRNA in whole cell lysates significantly potentiated its capacity to inhibit specific gene expression (Tuschl et al., 1999). In addition, using cell extracts from S2 cells subjected to RNAi, Hammond and coworkers described evidence for the presence of a nuclease that specifically degrades exogenous transcripts homologous to the dsRNA to RNAs of approximately 25 nt (Hammond et al., 2000). These observations suggest that a soluble enzymatic machinery exists whereby the initial dsRNA-mediated induction of RNAi leads to the establishment of RNAi in cells not directly exposed to dsRNA. We examined this induction process in the S2 transient transfection model using conditioned medium from a population of S2 cells exhibiting RNAi. Conditioned medium was collected and then added to a population of cells expressing the same transgene. The degree of inhibition seen in this second population of cells was consistent with the inhibition seen in stably expressing S2 cells transfected directly with dsRNA.

The specific down-regulation of gene expression in invertebrates could potentially have several therapeutic applications, for example, the use of dsRNA or dsRNA induced soluble factors to down-regulate genes involved in the parasitic infection of insect vectors. Given the ease with which heterologous genes can be expressed in *Drosophila* cell lines, assessing potential targets should now proceed relatively rapidly. In addition, studies of genetic mutants of Neurospora, *C. elegans* and *Drosophila* have begun to identify genes associated with

PTGS or RNAi, specifically an RNA-dependent RNA polymerase, a helicase, and several factors of no known biochemical function (Cogoni and Macino, 1999a,b; Fire, 1999; Jensen et al., 1999; Ketting et al., 1999; Tabara et al., 1999; Bosher and Labouesse, 2000). Despite the availability of such genetic systems, a model system with uniform populations of affected cells, as seen in this study, would be very useful. It should now be feasible to directly compare the endogenous gene expression of RNAi and non-RNAi populations, and by subtractive hybridization isolate genes associated with the mediation of RNAi in *Drosophila*. The potential scientific and medical applications of RNAi make it critical to continue investigation of the mechanism of RNAi, and the tissue culture model described here should be of significant use in this regard.

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References

Bahramian, M.B., Zarbl, H., 1999. Transcriptional and post-transcriptional silencing of rodent α1(I) collagen by a homologous transcriptionally self-silenced transgene. Mol. Cell. Biol. 19, 274–283.

Bosher, J.M., Labouesse, M., 2000. RNA interference, genetic wand and genetic watchdog. Nature Cell Biol. 2, E31–E36.

Bosher, J.M., Dufourcq, P., Sookhareea, S., Labouesse, M., 1999. RNA interference can target pre-mRNA. Consequences for gene expression in a *Caenorhabditis elegans* operon. Genetics 153, 1245–1256.

Caplen, N.J., Kinrade, E., Sorgi, F., Gao, X., Gruenert, D., Geddes, D., Coutelle, C., Huang, L., Alton, E.W.F.W., Williamson, R., 1995. In vitro liposome-mediated DNA transfection of epithelial cell lines using the cationic liposome DC-Chol/DOPE. Gene Therapy 2, 603–613.

Cherbas, L., Moss, R., Cherbas, P., 1994. Transformation techniques for *Drosophila* cell lines. In: Goldstein, L.S.B., Fyrberg, E.A. (Eds.), Methods in Cell Biology. Academic Press, San Diego.

Clemens, M.J., Elia, A., 1997. The double-stranded RNA-dependent protein kinase PKR: structure and function. J. Interferon Cytokine Res. 17, 503–524.

Cogoni, C., Macino, G., 1999a. Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. Nature 399, 166–169.

Cogoni, C., Macino, G., 1999b. Post-transcriptional gene silencing in *Neurospora* by a *RecQ* DNA helicase. Science 286, 2342–2344.

Fay, D.S., Stanley, H.M., Han, M., Wood, W.B., 1999. A *Caenorhabditis elegans* homologue of hunchback is required for late stages of development but not early embryonic patterning. Dev. Biol. 205, 240–253.

Felgner, J.H., Kumar, R., Sridhar, C.N., Wheeler, C.J., Tsai, Y.J., Border, R., Ramsey, P., Martin, M., Felgner, P.L., 1994. Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. J. Biol. Chem. 269, 2550–2561.

Fire, A., 1999. RNA-triggered gene silencing. Trends Genet. 15, 358–363.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391, 806–811.

Graham, F.L., Prevec, L., 1992. Adenovirus-based expression vectors and recombinant vaccines. Biotechnology 20, 363–390.

Hammond, S.M., Bernstein, E., Beach, D., Hannon, G.J., 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature 404, 293–296.

Jensen, S., Gassama, M.P., Heidmann, T., 1999. Taming of transposable elements by homology-dependent gene silencing. Nature Genet. 21, 209–212.

Kennerdell, J.R., Carthew, R.W., 1998. Use of dsRNA-mediated genetic interference to demonstrate that *frizzled* and *frizzled 2* act in the wingless pathway. Cell 95, 1017–1026.

Ketting, R.F., Haverkamp, T.H., van Luenen, H.G., Plasterk, R.H., 1999. *Mut-7* of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. Cell 99, 133–141.

Kwon, J.Y., Park, J.M., Gim, B.S., Han, S.J., Lee, J., Kim, Y.J., 1999. *Caenorhabditis elegans* mediator complexes are required for developmental-specific transcriptional activation. Proc. Natl. Acad. Sci. USA 96, 14990–14995.

Li, Y.X., Farrell, M.J., Liu, R., Mohanty, N., Kirby, M.L., 2000. Double-stranded RNA injection produces null phenotypes in zebrafish. Dev. Biol. 217, 394–405.

Lohmann, J.U., Endl, I., Bosch, T.C., 1999. Silencing of developmental genes in hydra. Dev. Biol. 214, 211–214.

Misquitta, L., Paterson, B.M., 1999. Targeted disruption of gene function in *Drosophila* by RNA interference (RNA-i): a role for *nautilus* in embryonic somatic muscle formation. Proc. Natl. Acad. Sci. USA 96, 1451–1456.

Montgomery, M.K., Xu, S., Fire, A., 1998. RNA as a target of double-stranded RNA-mediated genetic interference in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA 95, 15502–15507.

Ngo, H., Tschudi, C., Gull, K., Ullu, E., 1998. Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. Proc. Natl. Acad. Sci. USA 95, 14687–14692.

Palauqui, J.C., Balzergue, S., 1999. Activation of systemic acquired silencing by localised introduction of DNA. Curr. Biol. 9, 59–66.

Palauqui, J.C., Elmayan, T., Pollien, J.M., Vaucheret, H., 1997. Systemic acquired silencing, transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. EMBO J. 16, 4738–4745.

Sanchez Alvarado, A., Newmark, P.A., 1999. Double-stranded RNA specifically disrupts gene expression during planarian regeneration. Proc. Natl. Acad. Sci. USA 96, 5049–5054.

Schneider, I., 1972. Cell lines derived from late embryonic stages of *Drosophila melanogaster*. J. Embryol. Exp. Morphol. 27, 353–356.

Schneider, I., Blumenthal, A.B., 1978. *Drosophila* cell and tissue culture. In: Ashburner, M., Wright, T.R.F. (Eds.), The Genetics and Biology of *Drosophila*. Academic Press, London, pp. 265–315.

Tabara, H., Grishok, A., Mello, C.C., 1998. RNAi in *C. elegans*, soaking in the genome sequence. Science 282, 430–431.

Tabara, H., Sarkissian, M., Kelly, W.G., Fleenor, J., Grishok, A., Timmons, L., Fire, A., Mello, C.C., 1999. The *rde-1* gene, RNA interference and transposon silencing in *C. elegans*. Cell 99, 123–132.

Thomas, G.H., Elgin, S.C.R., 1988. The use of the gene encoding the α-amanitin-resistant subunit of RNA polymerase II as a selectable marker in cell transformation. Drosophila Information Service 67, 85.

Timmons, L., Fire, A., 1998. Specific interference by ingested dsRNA. Nature 395, 854

Tuschl, T., Zamore, P.D., Lehmann, R., Bartel, D.P., Sharp, P.A., 1999. Targeted mRNA degradation by double-stranded RNA in vitro. Genes Dev. 13, 3191–3197.

Wargelius, A., Ellingsen, S., Fjose, A., 1999. Double-stranded RNA induces specific developmental defects in zebrafish embryos. Biochem. Biophys. Res. Commun. 263, 156–161.

Wianny, F., Zernicka-Goetz, M., 2000. Specific interference with gene function by double-stranded RNA in early mouse development. Nature Cell Biol. 2, 70–75.

Wolffe, A.P., Matzke, M.A., 1999. Epigenetics: regulation through repression. Science 286, 481–486.